Chapter 164
Studying on Extraction of Betulin from the White Birch and Synthesis of Betulinic Acid

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Abstract Betulin and betulinic acid are natural product with antitumor and anti-HIV-1 activity. Betulin can be got by chemical extraction from a lot of plants. Betulinic acid can be synthesized from betulin. In order to get high biological activity compounds with antitumor and anti-HIV-1 activity from the bark of the white birch, the extraction and purification method of betulin was studied; the optimized extraction and purification condition was obtained. The two-step synthetic routes of betulinic acid from betulin were discussed. Betulinic acid was synthesized by oxidizing betulin with Jones reagent, followed by selective reduction with $NaBH_4$. IR, HPLC–MS, H^1-NMR were used to identify the structure of betulin and betulinic acid.

Keywords Betulin · Extraction · Betulinic acid · Synthesis

164.1 Introduction

Cancer and AIDS have been the most important diseases leading to death nowadays. But most of the commonly used medicines of anti-HIV or anticancer are proved to have many limitations in clinical application, especially toxic side effect and drug-resistance. Therefore, the development of medicine with perfect anticancer and anti-HIV effect is needed.

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Betulin exists in various plant resources, especially in the white birch bark. Betulin is a kind of bioactive substance with diverse biological activities, such as anti-inflammatory, antitumor, anti-HIV [1], which can be widely applied in food, cosmetic, and pharmaceutical fields. Betulin, as an important intermediate of medicine, can be obtained by extraction with organic solvent from the white birch bark [2].

Betulinic acid has antitumor, anti-HIV-1 and anti-inflammatory activities [3, 4], especially has an excellent effect on melanoma. As a potential precursor medicine of antitumor or anti-HIV, it has drawn the attention of researchers and investors. Betulin and betulinic acid have become a hotspot because of their excellent biological properties and good selectivity, low toxicity, and less side effects [5–8].

164.2 Materials and Methods

164.2.1 Materials, Reagents, Instruments

The white birch bark was collected in summer, Heilongjiang Province (2008–2009), in China. The birch bark was shattered to 60 meshes and immediately dried at 60 °C and stored in a dry and dark place.

Methanol, acetonitrile, ethanol, methanol, chloroform (Chl.), isopropyl alcohol (IPA), petroleum ether (B.P.: 60–90 °C, PE), ethyl acetate (EA), tetrahydrofuran (THF), and $NaBH_4$ are all of analytical grade.

H^1-NMR spectra are recorded on Bruker AM-400 NMR spectrometer in deuterated chloroform. HPLC spectra are recorded on Hitachi D–2000 spectrometers with UV detector. And Mass Detector is Waters 3,100; IR spectrometer is PerkinElmer spectrum65.

164.2.2 Methods

164.2.2.1 The Extraction and Purification of Betulin

The birch bark (100 g) with ethanol/IPA (10/1, V/V, 1,100 mL) was refluxed for 6 h in a Soxhlet's extractor; solvent was kept overnight at 0 °C. The precipitate was separated by filtration, washed on the filter with cold ethanol and then dried in vacuum drying oven.

In order to determine the optimum purification conditions for betulin, the solvents (A), solid–liquid ratio (B), and recrystallization times (C) were evaluated. And in every factor, we have chosen 3 levels to take the orthogonal design test (Table 164.1). Record every yield of crystal and determine the purity of betulin.

Table 164.1 Levels and factors in orthogonal design test of extraction of betulin

Level	Factor		
	A	B (V/V)	C
1	IPA/PE (10/1,V/V)	1/20	1
2	Chl./Methanol (1/1,V/V)	1/30	2
3	IPA/Methanol (3/1,V/V)	1/40	3

The optimal recrystallization process was determined based on both the yield of crystal (40 %) and the purity of betulin (60 %) as the combined value.

164.2.2.2 Synthesis and Purification of Betulinic Acid

A. Synthesis and Purification of Betulonic Acid

Freshly prepared Jones solution (2.3 mL) was added to a solution with betulin (1.0 g, 2.26 mmol) in acetone (100 mL) at 0 °C and the mixture were stirred at 0 °C. The course of the reaction was confirmed by TLC. After the reaction, the solvent was quenched with methanol (25 mL), stirred for 5 min, and added 40 mL H_2O. All the solid impurities in mixture were filtered out. After removing the organic solvent by reduced pressure distillation, the aqueous residue was extracted twice with ethyl acetate (40 mL). The organic layer was washed (H_2O, brine), dried ($MgSO_4$), and filtered. The solvent was removed in vacuum. The residue was column chromatographed over silica gel (200–300 mesh) using PE/EA (8/1) to obtain betulonic acid.

B. Synthesis and Purification of Betulinic Acid

$NaBH_4$ (0.227 g, 6 mmol) was gradually added to a solution with betulonic acid (0.90 g, 2 mmol) in THF (20 mL) at 0 °C and the mixture were stirred at 0 °C. The course of the reaction is confirmed by TLC. The reaction was quenched with HCl solution (3 mL, 2 mol/L). The solution was diluted with ethyl acetate (60 mL). The organic layer was washed (H_2O, brine), dried ($MgSO_4$), and filtered. The solvent was removed in vacuum to obtain crude betulinic acid. The crude betulinic acid was column chromatographed over silica gel (200–300 mesh) using PE/EA (6/1) to obtain betulinic acid.

164.2.2.3 Identification of the Chemical Structure and Purity of Betulin and Betulinic Acid

(1) Identification of the chemical structure

The chemical structure of betulin and betulinic acid was confirmed by IR, HPLC–MS,and H^1-NMR.

The HPLC analysis was carried out by VenusilC8 (4.6 × 250 mm). The mobile phase was methanol/acetonitrile/H_2O (45/45/10, V/V). The flow rate and injection volume were 1.0 mL/min and 10 μL. Betulin and betulinic acid were quantified, respectively, by UV detector at $\lambda = 210$ nm. All chromatographic operations were carried out at 30 °C [9, 10].

The MS conditions: The ion source is ESI (betulinic acid); the ion mode is negative; the scanning cover is 50–1000 u, the scanning mode is full scanning, the flow of desolvation gas is 700 L/h, the temperature of desolvation gas is 350 °C, the temperature of ion source is 150 °C.

(2) Determination of the purity of betulin and betulinic acid
A. The HPLC Conditions

The HPLC analysis was carried out by VenusilC8 (4.6 mm × 250 mm). The mobile phase was methanol/acetonitrile/H_2O (45/45/10, V/V). The flow rate and injection volume were 1.0 ml/min and 10 μL. Betulin and betulinic acid were quantified by UV detector at $\lambda = 210$ nm, respectively. All chromatographic operations were carried out at ambient temperature.

B. Preparation of Sample Solution

Accurately weighed equivalent to 2.0 mg of betulin or betulinic acid were dissolved with methanol/acetonitrile/H_2O (45/45/10, V/V, 1 mL). And then the solution was filtered through a membrane filter ($\varphi = 0.45$ μm).

C. The preparation of the blank reference solution

The blank reference solution were methanol/acetonitrile/H_2O (45/45/10, V/V) at the same conditions.

164.3 Results and Discussion

164.3.1 Results

164.3.1.1 The optimization process of the extraction and purification of betulin

The white birch bark (60 mesh, 100 g) with ethanol/IPA (10:1, V/V, 1100 mL) was refluxed for 6 h at 78 °C to give the crude betulin (34.7 g).

The orthogonal design test result of betulin purification is shown in Table 164.2.

According to the data (K1, K2, and K3) in Table 164.1, the effect of solvent, solid–liquid ratio, and recrystallization times on betulin quality were shown in Fig. 164.1.

Table 164.2 The result of the orthogonal design test of betulin purification

Ordinal	Factor			Result		
	A	B	C	Yield of crystal (%)	Purity (%)	Combined value (%)
1	1	1	1	57.5	54.18	55.51
2	1	2	2	38.9	89.02	68.97
3	1	3	3	20.8	96.65	66.31
4	2	1	2	40.4	71.04	58.78
5	2	2	3	27.2	95.60	68.24
6	2	3	1	63.4	67.02	64.37
7	3	1	3	20.2	96.40	65.92
8	3	2	1	71.7	66.38	68.51
9	3	3	2	45.1	76.24	63.78
K1	63.60 %	60.07 %	62.80 %			
K2	63.80 %	68.57 %	63.85 %			
K3	66.07 %	64.82 %	66.82 %			
R	2.47 %	8.50 %	4.03 %			

The combined value is derived from the yield of crystal (40 %) and the purity of betulin (60 %)

The optimum recrystallization condition is: the solvent is IPA/methanol (3/1, V/V), the solid–liquid ratio is 1/30 (g/mL) and the recrystallization time is 3 times.

164.3.1.2 The chemical structure and purity of betulin and betulinic acid

(1) The chemical structure of betulin and betulinic acid

The chemical structures of the betulinic acid and the betulin have been determined by H^1-NMR (Fig. 164.2a, b), respectively. IR (Fig. 164.3a, b) was used to identify the results. HPLC–MS spectrum of betulinic acid was carried out too (Fig. 164.4).

(2) The purity of betulin and betulinic acid

The HPLC spectrum showed that the purity of crude betulin is 50.56 % with a yield of 34.7 %. By the optimum recrystallization process, the purity of betulin is 95.2 % (Fig. 164.5a); yield of crystal is 20.8 %; and the purity of betulinic acid is 96.2 % (Fig. 164.5b) with a yield of 78.6 %.

164.3.2 Discussion

Betulin and betulinic acid possess antitumor, anti-HIV, and other biological properties. In the past decade, betulin, betulinic acid, and their derivatives have drawn the attention of researchers because of their excellent biological properties [11]. High quality betulin and betulinic acid have a huge market value.

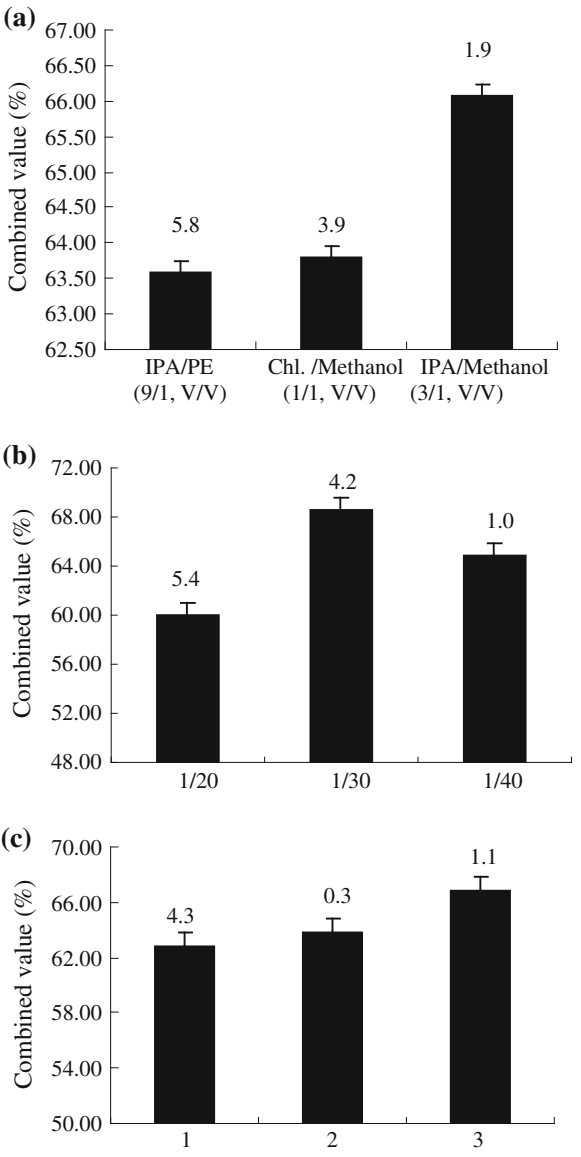

Fig. 164.1 The effect of the purification factor on betulin quality. **a** The effect of the solvent. **b** The effect of the solid–liquid ratio. **c** The effect of the recrystallization times. The combined value is derived from the yield of crystal (40 %) and the purity of betulin (60 %)

The commonly used organic-solvents, such as chloroform, methanol [12], methylbenzene [13], ethanol, dichloromethane, and diethyl ether [14], are ineffective for the extraction and purification of betulin. The experimental results showed: mixed-solvent ethanol/IPA is effective extraction solvent, which can improve the extraction yield as well as the purity of betulin. In this study, the orthogonal design test was used to optimize the recrystallization technology.

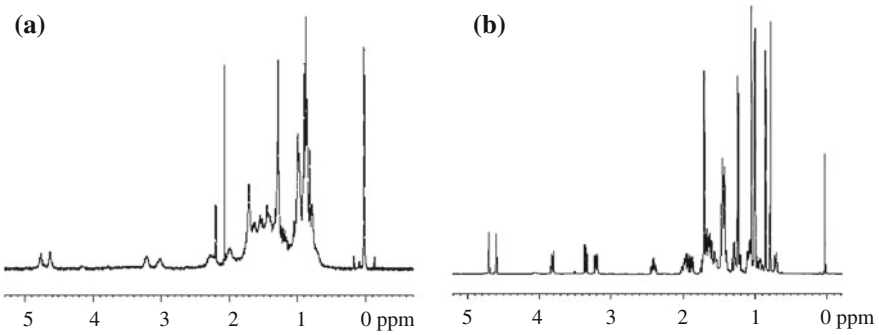

Fig. 164.2 **a** The H^1-NMR spectrum of betulinic acid. **b** The H^1-NMR spectrum of betulin

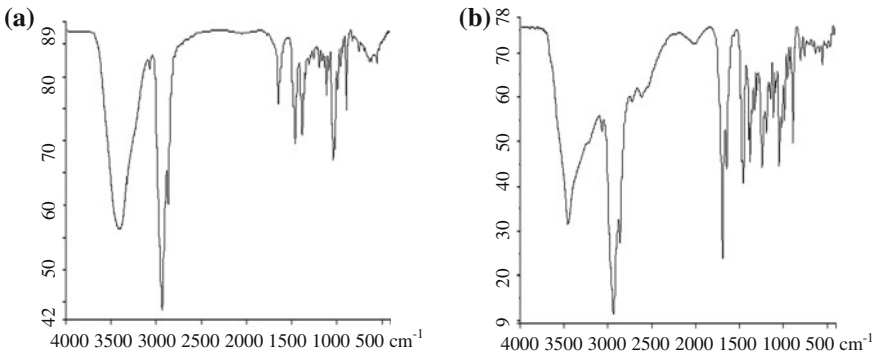

Fig. 164.3 **a** The IR spectrum of betulin. **b** The IR spectrum of betulinic acid

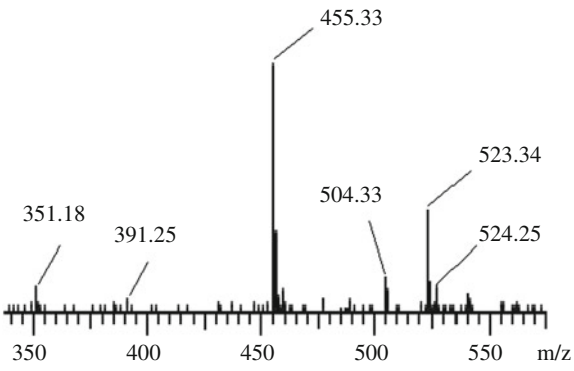

Fig. 164.4 The MS spectrum of betulinic acid

Betulinic acid can be obtained by extraction from plants and chemical synthesis [15–17]. But it is difficult to obtain betulinic acid by the method of extraction because of its very low content in plants [18]. Several kinds of synthesis methods

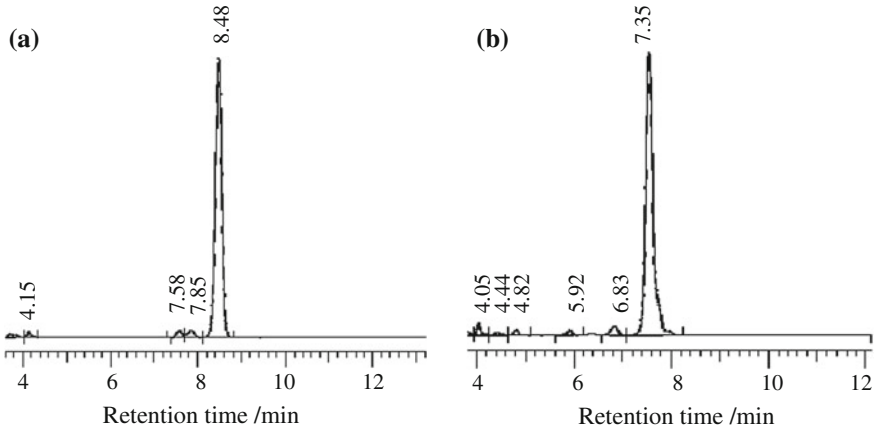

Fig. 164.5 **a** The HPLC spectrum of betulin. **b** The HPLC spectrum of betulinic acid

can be used to obtain betulinic acid [19, 20]. Semi-synthesis method by two-step synthesis with betulin as raw material is the most effective one. To this method, the betulin was oxidized by Jones reagent at first, followed by a reduction with $NaBH_4$ to afford crude betulinic acid of 73.4 % yield. The crude betulinic acid was purified by column chromatographed over silica gel (200–300 mesh) using PE/EA (6/1) to obtain 96.2 % betulinic acid (HPLC data) with a yield of 84.0 %.

164.4 Conclusion

Betulin was obtained from the white birch bark with ethanol/IPA as extraction solvent. Betulinic acid was prepared from betulin with two-step method. The optimal process and conditions were determined for the extraction of betulin and synthesis of betulinic acid. The betulin extraction method developed in this report may bring huge potential value for the development of new antitumor and anti-HIV-1 drug.

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